Applicant(s):	Matthew J. During	
Application No:	09/491,896	Group Art Unit: 1647
Filing Date:	January 24, 2000	Examiner: B. Bunner
Entitled:	Method for Modifying Target Receptor Function Associated With Neurological Disorders	
Atty. Docket No:	102194-6RCE	

RULE 132 DECLARATION OF DR. MATTHEW J. DURING

Dear Sir:

I, Matthew J. During, residing at 1512 Spruce Street, Apt. 2901, Philadelphia, PA 19102, hereby declare as follows:

1.   I am presently a Professor in the Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences at the University of Auckland, Private Bag, 92019, Auckland, New Zealand. I received an M.D. from the University of Auckland in 1982 and underwent training in internal medicine and endocrinology at the University of Auckland (1982-1986) and subsequently at Massachusetts General Hospital (1986-1989). I am board certified as an Endocrinologist in Australia and New Zealand, served as an attending physician on the endocrine service at Yale School of Medicine for several years, as well as, the Director of the Gene Therapy & Neurogenetics Laboratory at Yale University (1993-1996).

2.   My experience in this field also includes post-doctoral research at Harvard Medical School and Massachusetts Institute of Technology. For over ten years, I have conducted research in the fields of gene therapy and endocrinology. I established my own laboratory

in this field at the Yale School of Medicine, Thomas Jefferson University and presently continue this research at the University of Auckland.

3.   I am the inventor of the invention claimed in the above-identified U.S. Patent Application.

4.   I am familiar with the patent application at issue, and, through this declaration, I hope to address the Examiner's comments made in the May 5, 2003 and February 27, 2002 Office Actions relating to the election/restriction and the first paragraph enablement requirement.

5.   The claimed invention relates to a method for modifying the function of a target receptor associated with a neurological disorder in a subject by administering a vaccine comprising a therapeutically effective amount of an antigen, wherein the antigen elicits the production of antibodies in the circulatory system of the subject, or a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies bind to a target receptor on a neuronal cell in the central nervous system of the subject, and modify the function of the target receptor, such that modifying the function of the target receptor protects against a neurological disorder.

6.   To show that an antigen can be used to produce antibodies that provide protection against a neurological disorder, I submit a preliminary draft of a manuscript entitled "Protein Vaccination Leading to a Preconditioned Phenotype Associated with Resistance to Seizures and Neuroprotection" that demonstrates this concept. This work, described below, shows that protein vaccination with peptide antigens of an N-methyl-D-aspartate (NMDA) receptor lead to resistance of seizures and neuroprotection.

7.   The NMDA receptor consists of a heteromeric arrangement of NR1, NR2 and NR3 subunits. Recombinant proteins to two different functional NR1 regions of the NMDA receptor were used as antigens and tested in two rat models of epilepsy. The peptide antigens investigated were NR1[21-375] and NR1[654-800]. Both exerted anti-epileptic effects as defined by seizure latency and progression.

8.   The NR1[21-375] and NR1[654-800] peptides were expressed in bacterial cells and isolated from inclusion bodies. For vaccine preparation, the isolated NR1[21-375] and NR1[654-800] peptide antigens were dialyzed against phosphate buffer solution, concentrated to

5mg/ml, and then mixed with an equal volume of aluminium hydroxide adjuvant (Imject™ Alum; Pierce, Rockford, IL). All rats were vaccinated at 11-12 weeks of age. The dose of each vaccination was 0.1mg, administered as two intraperitoneal (i.p.) injections (0.2ml each). Two weeks later the dose was repeated as a single i.p. injection.

9.   Serum samples from the vaccinated rats were obtained 7 weeks post-vaccination and again at sacrifice. ELISA screening of the sera against their respective antigens demonstrated a strong humoral response had been generated in all vaccinated rats (Table 1). Both the NR1[21-375] and NR1[654-800] peptide antigens were found to be highly immunogenic, producing highly specific antibodies that recognized the native receptor. This demonstrated that antibodies to target receptors associated with a neurological disorder can be generated in the systemic circulatory system of a subject.

10.   Nine to twelve weeks after NR1[21-375] and NR1[654-800] peptide antigen immunization, subsets of each vaccination group (n=8-10) were subject to the kainate models of epilepsy. Two separate models of epilepsy were used for the study. In the first model, seizures were induced by systemic administration of kainic acid, while in the second model, kainic acid was administered directly into the hippocampus of the animals.

11.   The data from the first model shows that in the naïve (non-vaccinated) subgroup, systemic kainic acid administration induced progressive epileptic responses. However, the NR1[21-375] and NR1[654-800] peptide antigen vaccinated animals performed better than the non-vaccinated animals, although each vaccinated group of animals showed distinctly different anticonvulsant and neuroprotective phenotypes.

12.   In animals vaccinated with the NR1[21-375] peptide antigen, very few animals had seizures following induction of seizures by systemic kainate administration. In contrast, animals vaccinated with the NR1[600-854] peptide antigen developed seizure behaviors. However, progression through the defined stages of seizure was significantly delayed. Furthermore, analysis of brain sections, showed that the NR1[654-800] vaccinated animals had little or no neuronal loss in regions of the hippocampus.
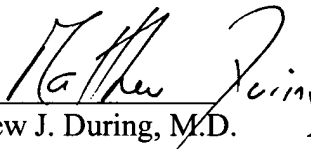
13.   Similar results were obtained using the second model of epilepsy, where the kainic acid was administered directly to the hippocampus and seizure activity recorded by measuring electroencephalograms of seizure activity (*See* Figure 1).

14.　Like the previous model, animals vaccinated with the NR1[21-375] peptide antigen (referred to as "NR1.R1R2 (H7)" and NR1.R1R2 (H8)" in Figure 1), had complete neuroprotection with little, or no signs of epilepsy. On the other hand, animals vaccinated with the NR1[654-800] peptide antigen (referred to as "NR1.2" and "NR1.extra2" in Figure 1) developed seizures at a rate slower than the non-vaccinated animals (referred to as "naïve" in Figure 1) and those animals vaccinated with a negative control peptide (referred to as "homer1a" in Figure 1). In addition, the vaccinated groups had fewer seizure episodes than controls.

15.　Collectively, this data demonstrates that vaccination of a subject with an antigen peptide vaccine against a brain antigen receptor provides neuroprotection against a neurological disorder by generating antibodies in the circulatory system of the subject that cross the blood-brain barrier upon insult or injury.

16.　I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated:　1/8/04

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Vaccination against distinct domains of the NMDA receptor leads to a preconditioned phenotype associated with resistance to seizures and neuroprotection.

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Abstract

The NMDA receptor is implicated in neuropathological states including stroke and epilepsy. We have recently described a genetic vaccine targeting the NR1 subunit of the receptor with prophylactic efficacy in both these conditions, mediated by the generation of NR1 autoantibodies (During et al., 2000). To further elucidate the mechanism of neuroprotection we have targeted distinct functional domains within NR1 in a modified vaccine approach. Recombinant proteins to two different functional NR1 regions were used as antigens and tested in two rat models of epilepsy, involving either systemic or intrahippocampal infusion of kainic acid. The antigens, corresponding to either NR1[21-375], the region containing the R1R2 domain, or NR1[654-800], the extracellular loop between TM3 and TM4 that contains the S2 lobe implicated in glycine binding, stimulated immunological responses which had anti-epileptic effects as defined by seizure latency and progression. Despite similar anti-epileptic efficacies, NR1[654-800] vaccine protected against hippocampal cell death, whereas NR1[21-375] vaccinated rats had neuronal damage typical of the naive rats. Both proteins showed similar antigenicity in inducing high titer antibodies, with no clear relationship between titer and neuroprotective (or anticonvulsant) action, suggestive of a threshold effect. Protein analysis of vaccinated rat brains showed a significant increase in expression of the neuroprotective proteins HSP70 and BDNF in NR1[654-800] animals, indicative of a preconditioned phenotype. These results further support the concept of targeted autoimmunity to protect the brain against insults, and isolate the S2 lobe of the NR1 subunit as a candidate target epitope.

Introduction

The N-methyl-D-aspartate (NMDA) receptor is one of three ionotropic receptor subtypes expressed in the mammalian CNS that along with the metabotropic glutamate receptors make up the glatamatergic receptor family (Chiu et al., 1999). Extensive studies implicate the receptor in a number of neuropathological states

including stroke, Parkinson's disease and epilepsy (Bittigau and Ikonomidou, 1997; Le and Lipton, 2001; Meldrum, 1995; Vajda, 2002).

Consisting of a heteromeric arrangement of NR1, NR2 and NR3 subunits (Cull-Candy et al., 2001; Ishii et al., 1993; McBain and Mayer, 1994), the NMDA receptor has binding sites for glycine and its agonist glutamate, as well as a redox site and sites for polyamines and divalent cations (Yamakura and Shimoji, 1999, review). In the rat brain the NR1 subunit is ubiquitously present in combination with the NR2A-D subunits, with the heteromeric makeup of individual receptor channels dependent on region and developmental stage (Ishii et al., 1993; Watanabe et al., 1994). The remaining NMDA receptor subunits, namely NR3A and the newly cloned NR3B (Chatterton et al., 2002; Nishi et al., 2001) are less well characterised though evidence points to a role in receptor modulation (Das et al., 1998) and regulation (Nishi et al., 2001) respectively.

Given the potential of NMDA antagonists to attenuate neuronal excitotoxicity, much has been invested in discovering and generating pharmacological agents for therapeutic use (Kohl and Dannhardt, 2001; Parsons, 2001). Previously we have described a novel NR1 vaccine with prophylactic efficacy in experimental stroke and epilepsy (During et al., 2000). The basis of this approach was the generation of autoantibodies to modulate NMDA receptor function. In the study we used an adeno-associated viral (AAV) vector to induce a polyclonal humoral response to the NR1 subunit. Epitope mapping of the vaccine sera suggested conserved epitopes in animals that showed neuroprotection. These epitopes lay within extracellular domains of NR1 such as the S1 and S2 domains, as well as the NH_2-terminal R1-R2 region. Both these domains are highly conserved in the NMDA receptor subunits and are involved in agonist binding, with S1-S2 containing the glycine binding site in the NR1 subunit (Armstrong et al., 1998; Ivanovic et al., 1998), and R1-R2 the spermine and ifenprodil sites (Masuko et al., 1999).

In this present study, we assessed these potentially functional epitopes in a protein based vaccine. To this end we designed and generated two truncated recombinant NR1 proteins each containing distinct functional domains: *NR1[21-375]* encompassing the R1-R2 domain and *NR1[654-800]*, consisting of the extracellular loop between TM3 and TM4 and containing the S2 lobe. These peptides were used as immunogens and tested in two variations of the kainic acid model of temporal lobe epilepsy. Our hypothesis was that these protein fragments would confer neuroprotection in a manner similar to that previously seen in the AAV-vaccinated animals (During et al., 2000). Analysis of the anti-epileptic phenotypes through protein profiling provided insights into the mechanisms of the observed protection, offering a platform for the design of future vaccines and related therapies of neurological disorders associated with overactivation of the NMDA receptor.

Materials and Methods

Animals. Male Wistar rats were housed in an animal care facility under a 12hr light-dark cycle with controlled humidity and temperature. Chow pellets and water was available *ad libidum*. All experiments were performed under University of Auckland Animal Ethics Committee (AEC) guidelines. During the course of this study the weights of the rats was monitored weekly.

Generation of recombinant NR1 proteins. For heterologous expression in bacteria, the regions encoding theaminoterminal subdomain of NR1 (NR1[21-375]) and the membrane-proximal portion of NR1 (NR1[654-800]) (Fig. 1A, 1B) were PCR-amplified from the mouse NR1 cDNA (During et al., 2000) using oligonucleotide primer pairs KL413(5'-ggtcgacatatgggatccgcctgcgaccccaagattgtcaacatcggc-gcggtg-3') / KL412(5'-gggatccaattgcggccgcttagtgatggtgatggtgatgatttgggatgacatgggtacca-3') and NR1ex2.fw(5'-aaggatccttcctggtgctggatcg-3') / NR1ex2.rev (5'-ccgaattcggttctcaaaagtgag-3'), respectively. The Homer1a control fragment was amplified from whole rat brain cDNA using primers and hom1-BI.fw (5'-

agggatccgggggaacaacctatcttcagc-3') and hom1.a-R1 (5'-ggaattcgtttaatcatgattgctgaa-3'). The NR1[21-375] BamHI/EcoRI fragment was inserted into the BamHI/MunI sites of pET3dR (containing a modified multiple cloning site in pET3 (Novagen, Madison, WI; K. Lehnert, unpublished), whereas the NR1[654-800] and homer1a BamHI/EcoRI fragments were inserted into the BamHI/EcoRI sites of pET3dR. These plasmids encode fusion proteins containing N-terminal Met-Gly-Ser extensions; the sequence of all expression plasmids was verified by DNA sequencing.

The recombinant NR1- and homer1a fragments were expressed in Escherichia coli BL21(DE3) according to standard protocols (Novagen pET System Manual). Inclusion bodies containing the recombinant proteins were isolated from cell lysates, washed by sonication and sedimented in 100mM NaH_2PO_4. 10mM Tris-Cl, pH 8.0. Purity and size of the recombinant proteins was assessed by SDS-PAGE in 12% reducing mini-slab gels under denaturing conditions.

Vaccinations. Washed inclusion bodies were solubilised and denatured in 8M urea and dialysed against PBS. The precipitated proteins were diluted to a final concentration of 0.5mg/ml, mixed with an equal volume of aluminium hydroxide adjuvant (Imject™ Alum; Pierce, Rockford, IL), and injected (i.p.) into rats 11 - 12 weeks of age. The dose of each vaccination was 0.1mg, administered as two separate injections (0.2ml each). Two weeks later the same dose was repeated in a single i.p. boost injection.

Blood sampling. Blood was taken from all vaccinated rats at three time points: prior to vaccination, 7 weeks post-vaccination, and at sacrifice. At the first two time points, 0.4 - 0.5mL samples were taken. At sacrifice, 8 - 9mL of blood was collected by heart puncture. Serum was obtained following coagulation and centrifugation (12,000g,10 min, RT) and stored at -20°C.

ELISA screening of immune sera. Aliquots of the antigen proteins were solubilised in 0.5% SDS and coated on 96-well MaxiSorp plates (Nunc, Denmark). After blocking, serum samples were applied to the plates in a 1:3 series dilution, and incubated overnight at 4°C. Bound IgG was detected with peroxidase conjugated secondary antibody (Santa Cruz, CA), and quantified at OD_{450} following addition of Turbo TMB-ELISA substrate (Pierce). Antibody titers were calculated by taking the inverse of the dilution at 50% saturation.

Whole brain membrane isolation and solubilisation. One half of a freshly dissected rat brain was homogenised in 15ml of 20mM Tris-HCl, pH 8.0 containing protease inhibitors (mini Complete, Roche, Germany) and centrifuged (800g, 20 min, 4°C) to remove whole cells and cellular debris. Following re-centrifugation (54,000g, 1 hr, 4°C) , the resulting membrane pellet was washed and resuspended in solubilisation buffer (20mM Tris-HCl, 1% TX-100, 5mM EGTA, 2mM EDTA, 1M NaCl, pH 8.0 containing protease inhibitors) and incubated for 2 hrs at 4°C. Insoluble matter was pelleted by centrifugation (100,000g, 30 min, 4°C) and the supernatant, termed 'solubilised whole brain membranes', was assayed for protein content using Biorad Protein Assay substrate (Biorad, Hercules, CA).

Antigen Capture ELISA. 96 well MaxiSorp plates were coated with monoclonal NR1 antibody (mAB363; Chemicon, Temecula, CA) at 0.5µg per well. Following blocking, freshly prepared solubilised whole brain membranes were applied, 15µg per well, and incubated overnight at 4°C. Sera was applied to the washed plates at 1:90 or 1:810 dilutions and incubated overnight at 4°C. As a control, affinity purified polyclonal NR1 antibody (Chemicon AB1516) was applied at the same dilutions. Detection of bound antibody utilised peroxidase-conjugated secondary antibodies and TMB substrate, as for standard ELISA screening, and OD at 450nm was determined.

Immunohistochemistry. Serum IgG was purified on immobilised Protein G as per manufacturer's instruction using ImmunoPure (G) IgG Isolation Kits (Pierce), and dialysed against PBS. Coronal hippocampal sections (35μm) were cut from a perfused (4% paraformaldehyde) naïve rat brain and prepared for immunohistochemistry as previously described (Mastakov et al., 2001). Polyclonal NR1 antibody (Chemicon AB1516; 1:200) or vaccine sera IgG (100μg/mL) was added and the sections were incubated overnight at RT. Bound IgG was detected with biotinylated anti-rabbit IgG or anti-rat IgG and ExtrAvidin peroxidase stained with DAB substrate.

Kainic acid induced seizure model: systemic administration. 9 - 12 weeks after immunization, rats received a single i.p. dose of kainic acid (KA, BioVectra, Charlottetown, Canada;10mg/kg). Seizure activity was monitored over a 90 min period and scored by a 'blinded' observer using the following scale modified from Racine et al., (1972): 0 = no response, 1 = immobility, staring, 2 = wet dog shakes (WDS), 3 = facial clonus (such as mastication), 4 = forearm clonus (unilateral or bilateral), 4.5 = bilateral forearm clonus with rearing and falling on side, 5 = forearm clonus with rearing and falling on back. Four days later, the animals were sacrificed. Blood was taken for serum analysis, and brains removed and frozen to -80°C.

Kainic acid induced seizure model: intrahippocampal administration. 17 - 21 weeks post-vaccination, further subgroups of vaccinated and naïve rats were tested for neuroprotection using an intrahippocampal kainic acid model of epilepsy. Under pentobarbital anaesthesia (72mg/kg, i.p.), bipolar recording electrodes (MS333-2B; Plastics One, Roanoke, VA) were stereotaxically implanted into the right hippocampus (AP-3.5mm, ML-2.4mm, DV-3.0mm from dura). In addition, a single guide cannula was positioned to rest on top of the dura directly above the left hippocampus (AP-3.5mm, ML-2.4mm). Both the electrode and the cannula were fixed with scull screws and dental acrylic cement. After a post-operative period of 1 week, kainic acid (0.04μg in 0.5μl) was administered via the cannula to an area 3mm below dura. EEG signals

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were recorded over a 2h period. The animals were then returned to their cages. After 4 days they were

sacrificed and tissues sampled as described above.

Statistical analysis. The percentage of vaccinated animals to progress through the described seizure stages

in the systemic kainic acid model of epilepsy was analysed using Chi square test and compared to non-

vaccinated animals. The same test was used to determine differences in the number of animals to develop

seizure activity in the intrahippocampal kainic acid model. Latencies to reach each stage for vaccinated and

non-vaccinated rats in both models were analysed with ANOVA followed by Fisher's PLSD test, as were the

number of seizure episodes. For all tests the significance level was 5%.

Terminal deoxynucleotidyl tranferase-mediated biotinylated-dATP nick-end labeling (TUNEL

staining). Following systemic kainic acid administration, 20μm coronal hippocampal sections were fixed,

washed and equilibrated in terminal deoxynucleotidyl tranferase (TdT) buffer (Invitrogen). Sections were

then incubated for 1 hr at 37°C in TdT buffer containing 40μM biotin-14-dATP (Invitrogen) and 150u/mL

recombinant terminal deoxynucleotidyl transferase rTdT. This was followed by brief incubation (15 min,

RT) in 2xSSC buffer (0.3M NaCl, 30mM Na citrate, pH 7.2) and a 10 min blocking step. Detection of

bound dATP was achieved by treating the sections with ExtrAvidin peroxidase (Sigma) and DAB substrate.

Immunoblot analysis of hippocampal extracts. Groups of rats were vaccinated as described above (n=5

per group) and sacrificed 9 days after the boost injection. Brains were removed and the hippocampus was

dissected. To obtain a crude lysate, tissue samples were sonicated in 10mM Tris-HCl, 2mM EDTA, pH 7.5,

containing protease inhibitors (mini Complete, Roche). The lysates were centrifuged (800g, 20 min, 4°C)

and the supernatants assayed for protein content. Aliquots were prepared for SDS-PAGE and stored -20°C.

20μg protein per tissue lysate underwent separation by SDS-PAGE (12% gels) and transfer onto

nitrocellulose membranes (Amersham Biosciences, UK). After blocking and probing with primary antibody

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(see below), bound antibody was detected with secondary antibody conjugated to horseradish peroxidase (Santa Cruz) and a chemiluminescent substrate (ECL Detection System, Amersham Biosciences). Each membrane was probed a total of three times. Between probes the membranes were washed and reblocked. Primary antibodies used included GAPDH (AbCam, England), PSD-95(Chemicon), ERK1/2, CREB, BDNF (Santa Cruz), HSP70 (Stressgene, Canada).

Chemiluminescent signals were captured on film (Hyperfilm ECL, Amersham Biosciences) and quantified using the Quantity One image analysis system (Biorad). Ratios were calculated for each protein signal with respect to GAPDH. Means and standard errors were determined over three separate blotting procedures, with analysis performed using ANOVA and Fisher's PLSD tests with a significance level of 2%.

Results

Generation of recombinant NR1 fragments. NR1[21-375] and NR1[654-800] were cloned into pET expression cassettes. Sequence analysis confirmed that the correct protein products were encoded. Once transformed with each of the pET constructs, positive BL21(D3) cell clones were amplified and induced to generate recombinant protein. Analysis of both the soluble and insoluble fractions by SDS-PAGE revealed all three recombinant proteins were expressed in the form of inclusion bodies.

Molecular size and purity of the recombinant proteins was assessed by SDS-PAGE analysis of the washed inclusion bodies (Fig. 1C). Both recombinant NR1 protein samples were shown to be >90% pure, with yields of 150mg and 80mg per 500ml culture for NR1[21-375] and NR1[654-800] respectively.

Homer1a is a neuronal scaffold protein enriched at post-synaptic sites. With a similar distribution pattern to NR1, Homer1a was chosen as a negative control antigen. It too was produced in a BL21(D3) cell line as inclusion bodies and isolated to >90% purity, with a yield of 40mg per 500ml culture.

In terms of size, all three proteins (NR1[21-375], NR1[654-800] and Homer 1a) were, upon SDS-PAGE migration, within 10% of their expected molecular weights.

Generation of NR1 antibodies. ELISA screening of the sera against their respective antigens demonstrated a strong humoral response in all vaccinated rats (Table 1). To verify whether the antigen-specific NR1 antibodies would recognise and bind native NR1, two complementary methods of screening were employed. In the first, native NR1 from solubilised whole brain membrane was captured with immobilised NR1 monoclonal antibody and presented to immune sera in an antigen capture ELISA. OD_{450} signal showed that the two different NR1 antigens had generated antibodies with an affinity for the native protein. Furthermore, the OD_{450} values for each serum assayed correlated with its antigen-specific titer (Table 2). The control Homer1a antisera did not recognise and bind to the captured native NR1.

In addition, IgGs from selected antisera were isolated and applied to naïve hippocampal sections. IgGs from NR1[21-375] and NR1[654-800] rats showed selective immunoreactivity to areas of the hippocampus expressing NR1 such as CA1 and CA3 neurons, as well as the dentate gyrus (Fig. 2). The pattern of binding was identical to that of an affinity-purified commercial NR1 polyclonal antibody. IgGs from pre-immune rat serum did not bind to the hippocampus. Homer1a antisera was shown to recognise native Homer protein by immunoblot screening against crude brain extract (not shown).

Neuroprotection in the kainic acid model of epilepsy. 9 - 12 weeks after vaccination, subgroups of rats were subjected to systemic administration of kainic acid (KA) to determine the neuroprotective efficacy of each vaccine antigen. In the naïve (non-vaccinated) subgroup, systemic kainic acid induced progressive epileptic responses. Of the 15 naïve rats tested, 11 (73.3%) progressed to stage 4, and of those, eight (53.3%) went on to stage 5. The mean latencies to reach each of these seizure stages were 64.2±2.1 and 69.5±2.2 min, respectively (Table 3). Of the vaccinated rats, the NR1[654-800] group differed significantly

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to naïve rats in seizure progression with an increased latency to reach stage 4 and stage 5 of 74.9±2.7 min

(*P<0.05*) and 83.0±1.3 min (*P=0.05*), respectively. In terms of the percentage of animals to progress

through each stage, both the NR1 vaccinated groups differed with respect to the naïve group. Of the nine

NR1[21-375] rats, three did not develop signs of seizure activity (*P<0.02*) and only one progressed to stage

5 (*P<0.05*). In the NR1[654-800] group only three of nine rats progressed to this final stage.

TUNEL staining of hippocampal sections 4 days after systemic kainate administration demonstrated

moderate neuronal cell death in the CA1 and CA3 regions as well as in the dentate gyrus and thalamus of all

treated naïve rats (Fig. 3, Table 4). Kainate-induced damage in NR1[21-375] vaccinated rats that developed

seizures was similar to that of naïve rats, yet in the NR1[654-800] rats that progressed to stage 4 and 5

seizures, neuronal damage was non-existent.

In another model of epilepsy, kainic acid was administered directly to the hippocampus of naïve and

vaccinated rats via unilateral injection. EEG signals were recorded in all treated rats. Representative

recordings are shown in Figure 4. From the EEG recordings it was possible to calculate the number of

seizure episodes over a 2 hr period, as well as latency to first episode and total ictal time (Table 5). For the

purpose of analysis, both control groups (naïve and Homer1a) were combined. The only significant

difference between either of the vaccinated groups and the controls was seen in the total ictal time for the

NR1[654-800] group (*P=0.05*). Although not significant, total ictal time in the NR1[21-375] group was also

reduced (*P=0.12*). In addition, two of the NR1[21-375] rats did not develop seizures (P =0.13) and mean

latency to first seizure episode for both the NR1[21-375] and NR1[654-800] rats was longer than for control

rats (10.7 and 11.3 min respectively, versus 8.3 min in the controls) Furthermore, the mean number of

seizure episodes in these two groups was smaller (10.4 for NR1[21-375] and 10.2 for NR1[654-800] versus

17.7 for controls; *P=0.07 and 0.09* respectively).

Immunoblot analysis of the vaccinated rat brains. For a subset of vaccinated animals, hippocampal lysates were prepared and probed nine days after boosting, when antibody titers were predicted to peak. Expression of several proteins associated with the NMDA receptor through signal transduction pathways, especially proteins with known neuroprotective properties, were investigated. Levels of PSD95, a component of the post synaptic density complex, were elevated in the hippocampus of NR1[654-800] animals (1.30-fold, *P<0.001*) and reduced in NR1[21-375] (0.61-fold, *P<0.001*). CREB expression was also increased in the NR1[654-800] group (1.28-fold, *P<0.02*). By contrast, ERK protein levels (ERK1 and ERK2) were unchanged in all vaccine groups. Of specific interest were the changes in heat shock protein 70 (HSP70) expression. Levels of this protein were 1.5-fold higher in the NR1[654-800] group (*P<0.001*), with no significant change in NR1[21-375] and Homer1a animals. BDNF expression was also examined. Similar to the profile for PSD95, expression of this protein was elevated in NR1[654-800] (1.8-fold more than naïve, *P<0.001*) but reduced in NR1[21-375].

Discussion

We have previously demonstrated that vaccine-generated autoantibodies to the NMDA receptor have prophylactic efficacy in animal models of stroke and temporal lobe epilepsy (During et al., 2000). In that study a genetic vaccine approach was used to target the entire NR1 subunit, yet it was noted that many of the protected animals had antibodies to specific epitopes on the receptor subunit. For this study it was decided to examine the importance of those epitopes as targets, and to determine whether our novel genetic vaccine approach could be generalized to a more traditional protein vaccine strategy.

Given that the epitopes of the earlier genetic vaccine study mapped to either the N-terminus of the NR1 subunit or the M3c or M4n regions of the S2 lobe, constructs were designed to incorporate these domains and recombinant proteins were generated in E. coli. Both NR1[21-375] and NR1[654-800] were found to be

highly immunogenic, producing specific antibodies that recognised the native receptor, with titers persisting throughout the course of the experiments.

Animals vaccinated with these antigens, along with a naive control group and a control group vaccinated with the intracellular scaffold protein Homer1a, were tested in two animal models of temporal lobe epilepsy. As expected, the NR1 vaccinated animals exhibited greater cerebral resistance than the non-vaccinated animals, although each NR1 group showed distinctly different anticonvulsant and neuroprotective phenotypes. Following systemic kainate administration very few NR1[21-375] animals progressed beyond a seizure rating of stage 4 . By contrast, NR1[654-800] animals developed seizure behaviours typical of the controls. Progression through the defined stages for this group however was significantly delayed. Furthermore, analysis of brain sections 3 days after seizure showed that NR1[654-800] animals that reached a seizure rating of 4 or greater had little or no neuronal loss in the CA1 or CA3 regions of the hippocampus, unlike the controls or the NR1[21-375] group.

In the intrahippocampal kainate model, different parameters were examined. Kainic acid was administered directly to the hippocampus via cannula and seizure activity recorded by EEG to provide a more sensitive measure of abnormal CNS activity. Like the previous model, only animals in the NR1[21-375] group showed complete protection (two of eight). Moreover, consistent with the systemic kainate model, we observed that NR1[654-800] animals developed seizures at a rate which was slower than that observed in the controls. Interestingly, both NR1 vaccine groups had fewer seizure episodes than controls.

It is apparent from the results obtained using both these models that there exists a dissociation between seizure activity and cell death or survival. A similar observation has been made with regard to the action of several antiepileptic drugs (AEDs). In 1989 Fariello et al described how the NMDA receptor antagonist MK-801 protected against kainate induced behavioural seizure activity and neuronal damage, yet did little to

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attenuate EEG seizure activity (Fariello et al., 1989). More recently, the protective actions of three different AEDs were analysed and compared (Maj et al., 1998). In that study, all three drugs protected susceptible neurons from damage following kainic acid. However, whereas two of the drugs (PNU-151774E and diazepam) were strongly anticonvulsant, lamotrigine showed only a trend towards the prevention of seizures. Regardless of its lack of robust anticonvulsant action, lamotrigine continues to attract interest as an antiepileptic drug, indicating the importance of neuroprotection alone in the development of AEDs.

Having ascertained anticonvulsant and neuroprotective properties in either NR1 antigen, we investigated possible mechanisms of action. Two hypotheses were considered. Following insult, protection could be attributed to the passage of antibodies across the blood brain barrier and receptor antagonism, a hypothesis put forward in our previous study (During et a., 2000). However, patch-clamp electrophysiological studies of primary hippocampal and mesencephalic neurons, using sera or IgG fractions from immunized animals, suggested no obvious acute effects on the NMDA channels (data not shown). Conversely therefore, chronic receptor modulation facilitated by allosteric changes associated with antibody binding could be involved. Evidence for this hypothesis lay with an observation in our earlier study that even under basal conditions vaccine-generated NR1 autoantibodies were present in the CSF. It has long been known that subthreshold, non-toxic overactivation of NMDA receptors can induce a tolerance to subsequent glutamate doses, even at otherwise lethal doses, a phenomenon termed preconditioning or ischemic tolerance (Kirino, 2002). We postulate that chronic exposure of NR1 auto-antibodies to the cell plasma membrane and membrane bound receptors results in receptor modulation and activation of signal pathways associated with preconditioning.

Pathways active in preconditioning involve mitogen-activated protein kinase (MAPK) (Shamloo et al., 1999; Skaper et al., 2001), calcium/calmodulin-dependent kinase (CaMK) (Shamloo et al., 2000) and other protein kinase pathways including protein kinase A and protein kinase C, all of which activate transcription factors within the nucleus to synthesise new proteins. Interestingly, hippocampal ERK1 and ERK2 levels,

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integral components of the MAPK pathway, were found to differ little between the vaccine groups and naïve

controls following vaccination. A similar observation was made for CaMKII and PKC (data not shown).

Despite this, expression levels of the transcription factor CREB, a common nuclear target of these pathways,

were significantly increased in the NR1[654-800] vaccinated animals. This observation suggests pathways

other than those mentioned above may be active in the NR1[654-800] vaccine-mediated preconditioned

phenotype. Activated CREB mediates the transcription of a host of genes, many of which have known

neuroprotective properties, including brain-derived neurotrophic factor (BDNF) (Biagini et al., 2001; Jiang

et al., 2003; Truettner et al., 2002; Yanamoto et al., 2000). As such, CREB expression and CREB activation

play pivotal roles in preconditioning (Mabuchi et al., 2001).

BDNF protein levels were also elevated in the NR1[654-800] group, further confirming induction of a stable

preconditioned cellular phenotype. In further support of the preconditioning hypothesis was an increase in

HSP70. Levels of the stress protein in animals vaccinated with NR1[654-800] were elevated by 50%

compared with controls and NR1[21-375] animals. Overexpression studies, both *in vivo* (Kelly et al., 2002;

Plumier et al., 1997; Rajdev et al., 2000) and *in vitro (Kelly et al., 2001)*, indicate a critical role of HSP70 in

the protection of neurons from cerebral ischemia and other forms of stress. Much is known of the roles HSPs

play in protein repair, refolding and trafficking within the cell, and it is through these actions that HSP70 is

likely to exert protective effects. Evidence also exists implicating antioxidant mechanisms in HSP70 action

(Polla et al., 1996).

In fragments NR1[21-375] and NR1[654-800] we have developed antigens with distinctly different

prophylactic potential. Not only have we demonstrated that NR1 protein vaccination can protect against

seizures, but have also explored the potential mechanisms. We now propose a more complex action of the

vaccine involving long-term interaction of the induced NR1 autoantibodies with the receptor, leading to

secondary or adaptive responses akin to preconditioning, as opposed to simple antibody-mediated receptor

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antagonism. The phenomenon of preconditioning has significant potential as a neuroprotective treatment for many disorders. However the acquisition of tolerance is dependent on new protein synthesis and modification, thus placing time limitations on single one-off treatments. The generation of circulating antibodies with the ability to continuously prime neurons prior to any insult removes these limitations, giving this prophylactic approach a distinct advantage over other therapeutic strategies.

Figure and Table Legend

Figure 1. Design and expression of the NR1 fragments. (A) Schematic representation of the NMDAR1 subunit and (B) the engineered and generated NR1 fragments [figure adapted from http://www.bris.ac.uk/Depts/Synaptic/info/glutamate.html]. (C) SDS-PAGE analysis of washed inclusion bodies from *E. coli* expressing each of the NR1 fragments, showing degree of purity and molecular weight prior to vaccination.

Table 1. Mean antibody titers. Sera were collected 7 weeks post vaccination and antigen-specific titers were determined.

* for the subgroup of animals tested in the intrahippocampal KA model, 18 weeks post vaccination, sera taken at time of sacrifice were also analysed for antibody levels.

Table 2. Specificity of vaccine sera for native NR1. OD at 450nm represents antibodies bound to immobilised NR1 protein in an antigen capture ELISA (see Methods).

• OD at 450nm for pre-immune sera shown in brackets.

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Figure 2. Immunohistochemical screening of vaccine antisera. Protein-G purified IgG from NR1 pre-immune or immune rat sera was used at 100µg/ml on naïve hippocampal sections. The affinity-purified commercial NR1 polyclonal antibody (Chemicon AB1516) was used at a 1:200. Note high intensity staining in areas known to highly express the NMDA receptor (CA1 and CA3 regions of the hippocampus and the dentate gyrus, DG). Scale bar 400 µm.

Table 3. Effects of the NR1 fragments as vaccine antigens in the systemic KA model of epilepsy. Data represent latency to reach each of the three defined seizure stages. Stage 2 = wet dog shakes , Stage 4= forearm clonus/wild running , Stage 5= forearm clonus accompanied by rearing and falling on back. SR = mean maximal seizure rating. Values represent mean +/- S.E.

- value in brackets represents number of rats to reach each of the stages.

[a] $P < 0.02$ with respect to naïve   [c] $P < 0.05$ with respect to NR1[21-375]

[b] $P < 0.05$ with respect to naïve   [d] $P < 0.05$ with respect to naïve

[e] $P = 0.05$ with respect to naïve

Figure 3. Seizure-induced damage in the hippocampus following systemic KA administration. TUNEL labelling (arrows) of neuronal cell death in the CA1 region of the hippocampus indicates the extent of damage in representative brain sections of NR1 vaccinated and control rats three days after receiving systemic kainic acid. Scale bar 200 µm.

Table 4. Seizure damage grading. The brains of rats to reach stage 4 or beyond in the systemic KA model were analysed by TUNEL labelling. Hippocampal injury was graded as follows: (-) no injury; (+) minimal injury (1-20 TUNEL labelled cells); (++) moderate (21-40 TUNEL cells); (+++) high (>40 TUNEL cells).

Table 5. Effects of the NR1 fragments as vaccine antigens in the intrahippocampal KA model of epilepsy. Data represent mean +/- S.E.

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- seizure activity is defined as continuous high frequency rhythmic spiking as recorded by EEG. Value in brackets represents number of rats to develop SE (>20 min continuous spiking).

[a] $P = 0.05$ with respect to control

Figure 4. Representative EEG recordings following intrahippocampal administration of KA. Kainic acid (0.04mg/ml) was injected into the left hippocampus of vaccinated and naïve rats via a guide canula. EEG readings were recorded over a 2h period. Scale bar 1 min..

Figure 5. Expression analysis of hippocampal lysates from vaccinated rats. (a) Representative immunoblots of hippocampal samples obtained from vaccinated rats. For each group, samples from 5 individual animals were screened three times for every antigen, standardised to GAPDH, and graphed as a function of relative expression to a non-vaccinated group (b). Values represent mean +/- S.E.

* $P < 0.001$ with respect to naïve

** $P < 0.01$ with respect to naïve

*** $P < 0.02$ with respect to naïve

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Table 1

	vaccination group			
	NR1.R1R2	NR1.2	NR1.extra2	homer
titer at week 7 (n = 20)	10082 ± 1092	10670 ± 985	23457 ± 1856	27174 ± 3258
titer at week 7 * (n = 6)	10376 ± 2502	9046 ± 2050	20535 ± 1768	30405 ± 6797
titer at week 18 * (n = 6)	5370 ± 1377	5712 ± 1471	8891 ± 1836	11586 ± 2886

Table 2

Vaccination group	rat	antigen-specific titer	OD @ 450nm	
			1:90	1:810
NR1.R1R2	H6	17556	0.432 (0.069)	0.185 (0.066)
	H3	8982	0.336 (0.066)	0.163 (0.069)
NR1.2	K2	23879	0.491 (0.069)	0.484 (0.074)
	K19	13194	0.461 (0.079)	0.345 (0.058)
NR1.extra2	J3	39149	0.516 (0.061)	0.364 (0.058)
	J15	23879	0.376 (0.057)	0.243 (0.065)
Homer1a	L6	41360	0.082 (0.062)	0.070 (0.062)
	L20	3729	0.068 (0.062)	0.066 (0.063)
AB1516			0.420	0.199

Table 3

| vaccination group | number of rats tested | WDS | latency to * | | SR (mean) |
			FC	FC/b	
naïve	15	25.7 ± 1.7 (15)	64.2 ± 2.1 (11)	69.5 ± 2.2 (8)	4.00 ± 0.34
NR1.R1R2	9	26.3 ± 3.9 (6[a])	64.3 ± 5.0 (6)	75.0 (1[e])	3.06 ± 0.58
NR1.2	10	26.2 ± 2.0 (10)	59.9 ± 2.5 (7)	71.1 ± 3.8 (6)	4.85 ± 0.11[f]
NR1.extra2	9	25.4 ± 1.9 (9)	74.9 ± 2.7 (7)[b, c, d]	83.0 ± 1.3 (3)	4.22 ± 0.34
Homer1a	8	25.4 ± 1.9 (8)	65.7 ± 2.8 (6)	75.3 ± 4.7 (4)	4.13 ± 0.47

[a] $P < 0.02$ with respect to naïve
[b] $P < 0.02$ with respect to naïve
[c] $P < 0.05$ with respect to NR1.R1R2
[d] $P < 0.002$ with respect to NR1.2
[e] $P < 0.05$ with respect to naïve
[f] $P < 0.01$ with respect to NR1.R1R2

Table 4

	Rat	Seizure rating	Minutes from first seizure to arrest	Neuronal damage				notes
				CA1	CA3	DG	thalamus	
NR1.R1R2	H3	4.5	6	-	-	-	-	
	H6	4.5	34	-	-	-	-	
	H13	4.5	15	+ +	+	-	-	
	H14	4.5	32	+ + +	-	-	+ + +	Left hemisphere only
	H15	5	31	-	-	-	-	
naive	I13	5	32	+ + +	+	-	+ + +	
	I21	4.5	33	-	-	-	-	
	I23	4.5	42	+ + +	+ +	+ +	+ + +	
	I24	4.5	27	-	-	-	-	
	I25	4.5	20	-	-	-	-	
	I27	4	12	-	-	-	-	
	I29	5	18	+ + +	-	+	+ +	
NR1.extra2	J1	5	24	+	-	+ +	-	
	J2	4.5	18	-	-	-	-	
	J3	4.5	6	-	-	-	-	
	J6	4.5	15	-	-	-	-	
	J14	5	17	-	-	-	-	
	J15	5	23	-	-	-	-	
	J16	4.5	3	-	-	-	-	
NR1.2	K1	4	26	+ + +	+	-	+ + +	
	K2	5	42	+ + + +	+ +	+ +	+ + + +	
	K3	5	31	+ + + +	+ +	+ +	+ +	
	K5	5	22	+	+	+ +	+ +	
	K6	5	18	-	-	-	-	
	K16	5	37	+ +	-	+	+ +	

Table 5. Effects of the NR1 fragments as vaccine antigens in the intrahippocampal KA model of epilepsy. Data represent mean +/- S.E.
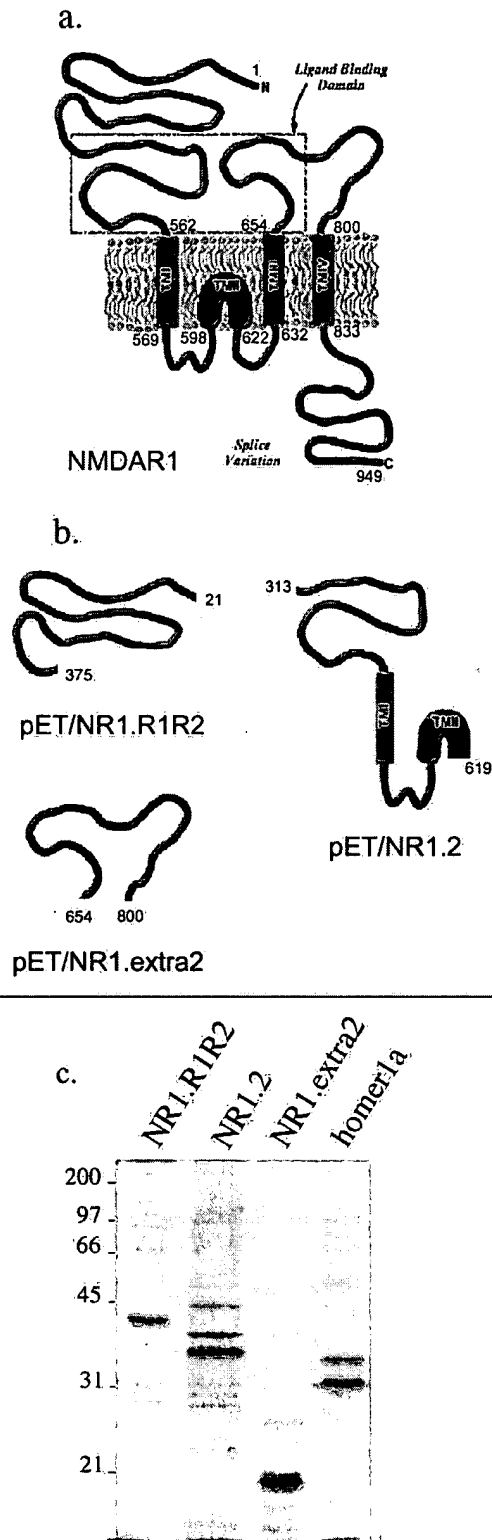
seizure activity is defined as continuous high frequency rhythmic spiking as recorded by EEG. Value in brackets represents number of rats to develop SE (>20 min continuous spiking).

[a] P = 0.05 with respect to control

vaccination group	number of rats tested	number to develop seizure activity *	latency to first seizure episode ^ (min)	number of episodes	total ictal time (min)
naïve	5	5 (1)	8.7 ± 1.9	15.4 ± 3.4	24.3 ± 7.8
NR1.R1R2	8	6 (1)	10.7 ± 2.8	10.4 ± 3.3	15.0 ± 5.6
NR1.2	6	5 (1)	8.1 ± 1.9	18.2 ± 3.9	22.9 ± 5.9
NR1.extra2	6	6 (0)	11.3 ± 1.2	10.2 ± 2.1	10.5 ± 2.4[a]
Homer1a	4	4 (0)	7.7 ± 1.1	20.5 ± 4.2	30.6 ± 10.8

[a] P < 0.05 with respect to Homer1a

1268524.1

FIGURE 1

a.

Ligand Binding Domain

1
N

562   654   800

569   598   622   632   833

NMDAR1   Splice Variation   C
949

b.

21

375

pET/NR1.R1R2

313

619

pET/NR1.2

654   800

pET/NR1.extra2

c.

200
97
66

45

31

21

FIGURE 3

Controls

NR1.R1R2

NR1.2

NR1.extra2

naive (stage 5, 77')

homer (stage 5, 70')

H14 left (stage 4.5, 58')

H15 (stage 5, 75')

H14 right

K5 (stage 5, 79')

K1 (stage 4, 64')

J1 (stage 5, 70')

J6 (stage 4.5, 75')

1297600.1

naive

NR1.R1R2 (H7)

NR1.R1R2 (H8)

NR1.2

NR1.extra2

homer1n

NR1.R1R2   NR1.2

NR1.extra2   naive

AB1516

FIGURE 4